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Occurrence of a kinin-like peptide in the urinary bladder of the toad *Bufo marinus paracnemis* Lutz*

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PREVIOUS work,[1] utilizing a preparation *in vitro*[2] of the urinary bladder of the toad, has shown that bradykinin although inactive itself upon the osmotic water flow through the bladder wall, when added to the preparation with vasopressin, inhibited the permeability-increasing effects of the hormone. The same reversible antagonism was also observed with kallidin, eledoisin, and physalaemin,[3] and has been shown to occur in the toad skin as well.[4] These data, added to the fact that kinins occur in the skin of amphibians,[5,6] suggested a search for kinins in the bladder tissue.

Materials and Methods

Animals. The toads (*Bufo marinus paracnemis* Lutz) were killed immediately after being captured. Their weight varied from 200 to 750 g.

Extraction procedure. After being quickly excised, the bladders were kept at 0° until the last animal was killed. The pool of bladders was minced with scissors and weighed. The fresh bladder tissues were extracted with 10 vol. (w/v) of 99% methanol according to the method of Anastasi *et al.*,[5] glacial acetic acid being added to adjust the pH of the mixture to 3·5. The bladders were then extracted at a temperature of 4–5° for a week. The liquid was decanted and the tissues were again extracted with 5 vol. (w/v) of 99% methanol and acidified to pH 3·5 with glacial acetic acid for another week at 4–5°. Thereafter, the second liquid was also decanted and added to the first extraction liquid. The whole mixture was filtered down and then concentrated by evaporation under reduced pressure to a volume ten times smaller. Crude methanol extracts were free of fat-soluble contaminants in a separatory funnel after three treatments with 3 vol. (v/v) of petroleum ether. The active material, collected in the aqueous phases, was evaporated under reduced pressure and resuspended either in saline solutions (for pharmacologic characterization) or in 0·1 M acetic acid (for chromatographic studies).

Biological assays. Pharmacological assays of the defatted extract were always carried out in the presence of atropine and diphenhydramine occasionally also in the presence of a serotonin inhibitor, brom-lysergic acid diethylamide (BOL). In order to characterize its activity, the extract was applied both to preparations of *perfused* isolated segments of smooth muscles as well as to *superfused* organs. Perfusion assays were carried out on the guinea pig ileum, the rat duodenum, and the rabbit duodenum, bathed with Tyrode's solution, and on the rat uterus perfused with de Jalon's solution. The extract was also assayed upon segments of cat jejunum, rat colon and rat stomach superfused with Krebs solution.[7]

The extract was also assayed on the isolated urinary bladder of the toad[2] suspended in a bath of Ringer's solution and filled with a diluted Ringer so that an osmotic gradient was built up across the bladder wall. Under these conditions water moves down the gradient, across the bladder and out of it. This loss of water by the bladder can be easily estimated gravimetrically, by recording the weight of the bladder before and after a given time interval. Neurohypophysial peptides increase the permeability of the bladder wall, thus stimulating the osmotic water loss. It has been shown that the kinins antagonize the permeability-increasing effects of the pituitary hormones.[3] The bladder extract was applied to the bladder preparation in the presence of oxytocin, and the effect was compared to that of the synthetic bradykinin. A detailed description of the technique has appeared elsewhere.[3]

The systemic blood pressure of the dog and the cat was measured in the femoral artery by means of a mercury manometer and recorded on a kymograph. The extract as well as synthetic bradykinin was injected intravenously and their effects were compared.

Chemical studies. Paper electrophoresis was performed on Whatman 3 MM paper with 0·1 M sodium acetate (pH 5·6) for 7 hr. Electrophoretic runs were carried out on bladder extracts, bradykinin, and

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eledoisin in simultaneous experiments. When assaying the eluates from the electrophoretic paper strips, the isolated and perfused segments of guinea pig ileum, cat jejunum, and rat duodenum were used as the assaying organs.[6,7]

Further purification of the extract was achieved by gel filtration through Sephadex G-25 and Bio-Gel P-2 columns.

Sephadex G-25 columns (2·4 × 240 cm). The column was filled with 150 g of the gel and eluted with 0·1 M acetic acid (pH 3·5). Five ml of a concentrated solution of bladder extract (equivalent to active material derived from 3·5 g of fresh bladder tissue) was then applied to the column and 10-ml fractions were collected at 15-min intervals. The absorbancy at 280 nm was recorded for all of the fractions, which were then assayed on the guinea pig ileum and the rat duodenum (Fig. 1). The fractions that relaxed the duodenum were lyophilized, and the samples from the various experiments were pooled together. This pool was then applied to a column of Bio-Gel P-2.

Bio-Gel P-2 columns (2·2 × 55 cm). The column was charged with 40 g of the gel and eluted with 0·1 M acetic acid (pH 3·5). Two ml of a solution containing the active pool from the Sephadex G-25 columns was then applied to the column and 3-ml fractions were collected at 15-min intervals. These fractions were analyzed for absorbancy at 280 nm and assayed as above (Fig. 2).

Bladder homogenates. Ten toads, weighing 400–500 g, were double-pithed and had their urinary bladders quickly excised and then weighed. They were suspended in 4 vol. of ice-cold TBS solution,*

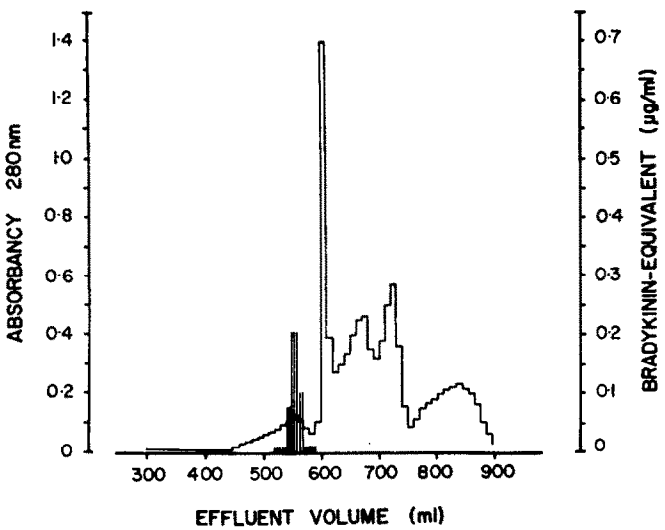

FIG. 1. Elution pattern of the bladder extract when applied to a column of Sephadex G-25 (2·4 × 240 cm) utilizing 0·1 M acetic acid (pH 3·5) as eluent. Flow rate: 10 ml/tube/15 min. The spasmogenic activity of the fractions was assayed on the isolated guinea pig ileum. The active fractions were then assayed on the rat duodenum and those exhibiting a relaxant effect were pooled together. The hatched area shows the spasmogenic action of the pooled fractions upon the guinea pig ileum, expressed in terms of activity comparable to that of a bradykinin standard solution in the same preparation (bradykinin-equivalent). Abscissae: effluent volume (ml). Ordinates: absorbancy at 280 nm (left) and bradykinin-equivalent in µg/ml (right).

and homogenized in a small blade homogenizer at 15,000 rev/min for 2 min. The homogenate was centrifuged at 10,000 rev/min for 10 min in a refrigerated centrifuge (MSE High Speed 25). The supernatant was used for the assay of kinin-forming and -destroying activities.

Kininase activity. An adequate amount of enzymic preparation was added to a siliconized glass tube (E) containing 2·0 µg bradykinin in buffered solution (pH 7·5). The tube was kept in a crushed ice bath. Immediately after completing the mixture, an aliquot was pipetted out and applied to a perfused segment of guinea pig ileum in order to obtain a kymographic recording of the kinin activity

* TBS solution's composition[8] per liter: NaCl, 7·5 g; $CaCl_2$, 0·02 g; $MgCl_2$–6 H_2O, 0·1 g; 1 N HCl 18 ml; triethanolamine, 2·8 ml; pH 7·5.

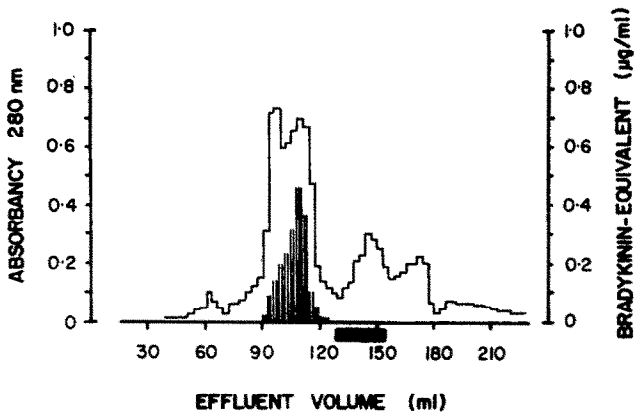

FIG. 2. Gel filtration in Bio-Gel P-2. The fraction pool (see text) obtained from a Sephadex G-25 chromatography was applied to a Bio-Gel P-2 column (2·2 × 55 cm) charged with 0·1 M acetic acid (pH 3·5). Flow rate: 3 ml/tube/15 min. The assay of activity of the fractions on the guinea pig ileum and the rat duodenum preparations proceeded as in Fig. 5. The hatched area shows the spasmogenic activity upon the guinea pig ileum of the eluate fractions that also relaxed the rat duodenum. Also shown is the position (horizontal bar) of bradykinin when eluted in the same column of Bio-Gel P-2, under the same experimental conditions, in a successive experiment. Legends as in Fig. 1.

at zero time. The mixture was then put in a water bath at 37° for incubation. Thereafter, at incubation times of 6, 12 and 18 min, aliquots of the same volume pipetted at zero time were applied to the ileum preparation. A control tube (C) containing 2·0 μg bradykinin in buffered solution (pH 7·5) was simultaneously prepared and treated as for tube E. Thus, at the same time intervals and alternating with the tube E samples, aliquots of tube C were added to the ileum preparation in volumes that were

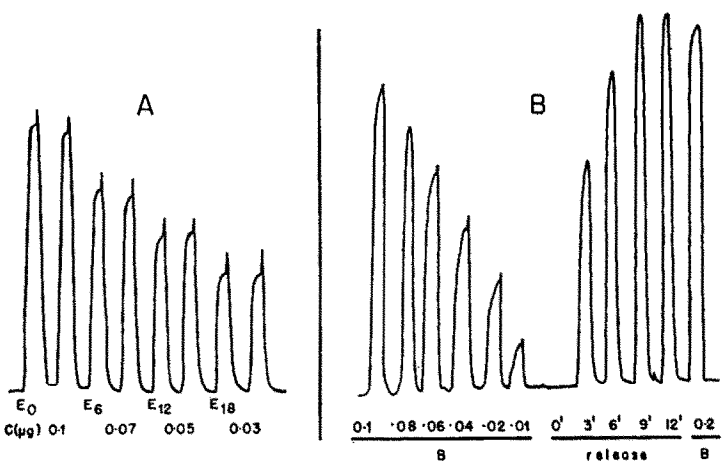

FIG. 3.(a) Kininolytic activity of bladder homogenate assayed on the guinea pig ileum as described in Methods. Each milliliter of homogenate contained 75 μg protein. E = additions of homogenate-containing mixture at different time intervals (0, 6, 12 and 18 min). C = aliquots of control mixture containing only bradykinin and added at the same incubation times. (b) Assay of the kinin-forming activity of bladder tissue upon the guinea pig ileum. An enzymic preparation containing 0·75 mg protein was diluted with distilled water (1 : 2) at pH 5·0 for activation. When incubated with Horton's substrate in the presence of 1,10-phenanthroline, it released a smooth-muscle contracting substance that accumulated progressively in the reaction mixture for the first 10 min to level off thereafter. B = synthetic bradykinin (μg). Proteins were measured using a biuret method.[9]

adjusted so as to exhibit a control spasmogenic response which would as far as possible match that obtained immediately before from tube E (Fig. 3a).

Kininogenase activity. Kininases present in the bladder tissue would inactivate the kinin released; therefore, to detect kinin-forming activity, it was necessary to use an inhibitor of the kininase of the bladder, namely 1,10-phenanthroline.[4] In addition, bladder tissue was also found to have little free kininogenase activity. However, it has apparently large amounts of prekininogenase that could be activated by acid and dilution. To test kinin release in siliconized tubes, 0·5 ml of the enzymic preparation was mixed with 0·5 ml of distilled water, the pH was adjusted to 5·0 and the mixture was incubated at 37° for 30 min. Then, with the mixture kept in a crushed ice bath, phenanthroline was added to a concentration of 10^{-3} M, the pH was again adjusted to 7·5, 0·5 ml of Horton's substrate[10] was added; and a final volume of 2·0 ml was completed with TBS solution. An aliquot was immediately pipetted out and applied to a perfused guinea pig ileum for the zero time activity. The mixture was then put in a water bath at 37° for incubation. Thereafter, at the indicated incubation times, aliquots of the same amount pipetted at zero time were applied to the ileum preparation.

Drugs. The following drugs were used: Bio-Gel P-2 (Bio Rad); bradykinin ("BRS-640", Sandoz, No.64052); chymotrypsin, 3 times crystallized, salt-free (Mann); 2,2′-dipyridyl (Sigma); eledoisin ("ELD-950" Sandoz, No. 64001); oxytocin ("Syntocinon", Sandoz, Br 88675); 1,10-phenanthroline (Merck Darmstadt); Sephadex G-25 (Pharmacia); trypsin, 2 times crystallized, salt-free (Mann).

Results

Pharmacologic characterization. The aqueous crude extract of bladders was found to contract most of the smooth muscle preparations except for those of the rat duodenum. Its spasmogenic action was observed upon the following preparations: guinea pig ileum, rat uterus, rabbit duodenum, rat stomach, and the cat jejunum. Both the guinea pig ileum and the cat jejunum exhibited a good dose–response relationship, the cat jejunum preparation (Fig. 4) being more sensitive to the extract. On the other hand, as Fig. 5 shows, the rat duodenum was relaxed by the extract in a way similar to bradykinin. When injected intravenously in the anesthetized dog and cat, the bladder extract caused hypotensive responses, showing a good dose–response correlation (Fig. 6).

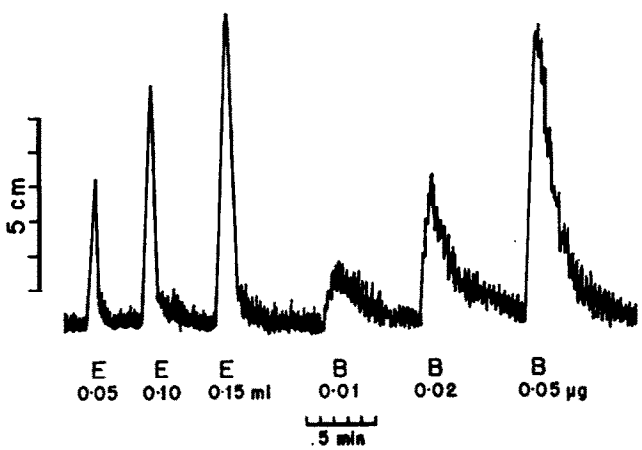

FIG. 4. Cat jejunum strip superfused with Krebs solution. Response to synthetic bradykinin (B) and to the bladder extract (E). Each milliliter of the extract solution contained activity derived from 66·88 mg of fresh bladder tissue.

In previous work, it was shown that the increase in the transepithelial osmotic water flux evoked by neurohypophysial hormones, when applied to the bladder preparation, was inhibited by bradykinin as well as by other kinins.[3] The bladder extract itself was also inactive when applied alone to the preparation *in vitro* of the isolated toad bladder. However, like the kinins, when added to the preparation together with oxytocin, the bladder extract markedly inhibited the permeability-increasing effects of the neurohypophysial peptide (Fig. 7). Figure 7 shows that the extract caused a parallel shift of the log-dose–response curve for oxytocin in such a way that in order to obtain the same response in the presence of that amount of extract it was necessary to use an oxytocin dose about 25 times larger than the control dose. For a better estimate of the sensibility of the bladder tissue to the active material of

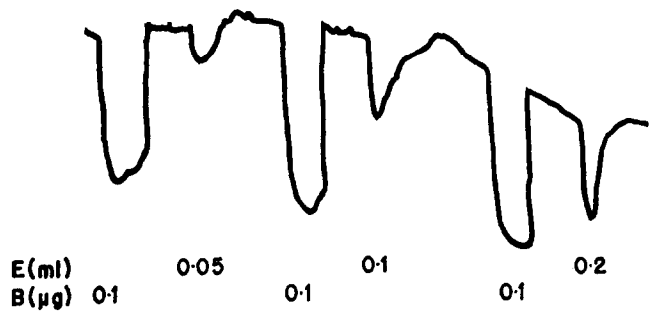

FIG. 5. Responses of isolated rat duodenum perfused with de Jalon's solution to synthetic bradykinin (B) and to the bladder extract (E). Each milliliter of the extract solution contained active material derived from 0·40 g of fresh bladder tissue.

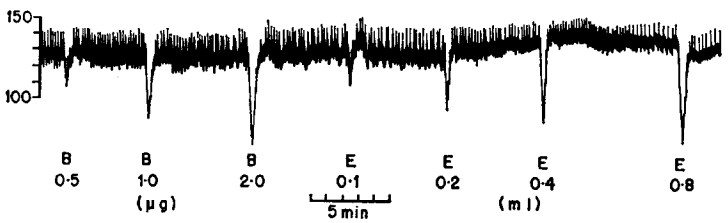

FIG. 6. Blood pressure recording (mm Hg) of a dog anesthetized with pentobarbital. Responses to the intravenous administration of different doses of bradykinin (B) and bladder extract (E). Each milliliter of the extract solution contained active material derived from 0·27 g of fresh bladder tissue.

the bladder extract, it should be noted that the same amount of extract that brought about that marked inhibition of the oxytocin effects on the bladder preparation caused a hypotension in the dog (Fig. 6) equivalent to the hypotensive response to 1·0 μg bradykinin. However, in the bladder preparation, 1·0 μg/ml of bradykinin caused an inhibition of the effects of oxytocin about 5 times smaller than the one obtained with the extract.

Chemical studies. Paper electrophoresis, in simultaneous experiments, of aliquots of synthetic bradykinin and eledoisin, as well as of bladder extract, showed that the bladder peptide migrated toward the anode, while bradykinin and eledoisin migrated toward the cathode. Figure 8 depicts the mobility of each peptide and of the extract. When assaying the eluates from the electrophoretic paper strips, the isolated and perfused segments of guinea pig ileum, cat jejunum, and rat duodenum were used as the assay organs.[6,7] The eluates from bladder extract strips contracted both the guinea pig ileum and the cat jejunum, while relaxing the rat duodenum muscle.

Further purification of the extract was achieved on gel filtration chromatography (see Methods). From the Sephadex G-25 columns (Fig. 1), only the fractions that relaxed the rat duodenum were collected to form the pool of activity that was subsequently chromatographed on the Bio-Gel P-2 columns (Fig. 2). Figures 1 and 2 show the elution pattern of the bladder extract when applied to the columns of Sephadex G-25 and Bio-Gel P-2 respectively.

Enzymic hydrolysis. The action of proteolytic enzymes upon the extract was tested in order to characterize its nature. The procedure was in essence the same one described for the kininolytic assay (see Methods). The amount of extract used was one that contained active material derived from 150 mg of fresh bladder tissue. To this amount of extract, 1·0 mg of trypsin was added, and in other experiments 100 μg chymotrypsin was added instead. The buffered mixture (pH 7·8) was then assayed upon a desensibilized guinea pig ileum preparation. The extract was not hydrolyzed by trypsin, and its activity was even increased after tryptic treatment. Chymotrypsin, on the other hand, fully hydrolyzed the extract in about 60 min under the present experimental conditions.

Kininolytic activity of the bladder tissue. Buffered bladder homogenates (pH 7·5) when incubated with bradykinin inactivated the peptide (Fig. 3a). Better characterization and purification of the enzyme were not done; however, preliminary studies showed it to be most active between pH 7·5 and

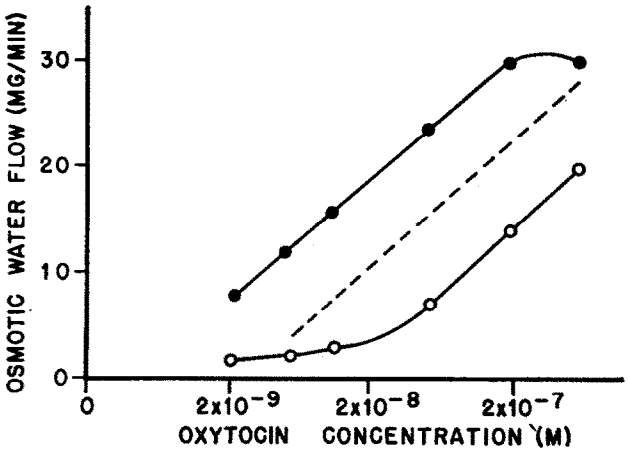

FIG. 7. Oxytocin influence on the water permeability of the isolated urinary bladder of the toad. Log-dose–response curves for oxytocin in the absence (●) and in the presence (O) of 0·25 ml of a solution of bladder extract. Each milliliter of this solution contained activity derived from 0·27 g fresh bladder tissue. Each point is the mean of 3 determinations. The interrupted straight line shows the response to oxytocin in the presence of 1×10^{-6} M bradykinin (1 μg/ml).[3] The substances were always added to the serosal Ringer's solution for 20 min.

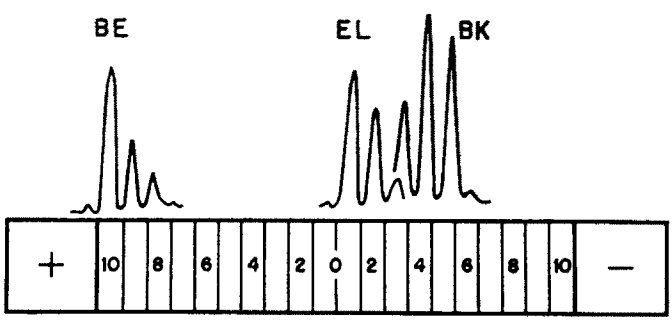

FIG. 8. Diagrammatic representation of the results of paper electrophoresis as assayed on the guinea pig ileum. The amount of substances used was: 60 μg bradykinin (BK) in 60 μl, 10 μg eledoisin (EL) in 100 μl, and 120 μl of bladder extract (BE). Each milliliter of the solution of bladder extract contained active material derived from 0·27 g of fresh bladder tissue. O = origin.

8·0. In the presence of inhibitors, this kininase behaved like that found in erythrocytes[11] and in squamous epithelial cells of the oral cavity.[12] Among the chelating agents only 1,10-phenanthroline and 2,2′-dipyridyl inhibited the kininase of the bladder.[4]

Kinin-releasing activity.[13] Figure 3 also shows the kininogenase activity of the bladder tissue after previous activation by acid and dilution. Control experiments showed that there was no spontaneous release of kinins when Horton's substrate or the bladder homogenate was separately incubated with the kininase inhibitor. Furthermore, it has been observed that the bladder homogenate also displayed esterase activity upon benzoylarginine ethyl ester (BAEE).

Discussion

The present data demonstrate that the urinary bladder of the toad *Bufo marinus paracnemis* Lutz contains a hypotensive polypeptide with the characteristics of a kinin-hormone.[14] Like bradykinin, the bladder peptide was also resistant to trypsin hydrolysis, and it was destroyed by chymotryptic digestion. Pharmacologically, the bladder peptide behaved similarly to bradykinin, thus contracting conspicuously the cat jejunum preparation and relaxing the rat duodenum muscle. The outstanding

difference was the exquisite sensibility of the isolated urinary bladder of the toad to the bladder extract, as compared to bradykinin, this assay organ being about 50 times more sensitive than the dog arterial blood pressure preparation. Paper electrophoresis showed that bradykinin and the active peptide from the bladder are distinctly charged and consequently migrated toward opposing poles.

This bradykinin-like peptide apparently has a molecular weight of 1000 or slightly above, judging from the elution patterns obtained from the Bio-Gel P-2 columns both for the extract and bradykinin (the latter's molecular weight is 1000). Although its structure has not as yet been elucidated, a proper name for this substance would be bufokinin.

Because of their spasmogenic action upon the rat duodenum, eledoisin-like peptides are distinguished from bradykinin-like compounds.[6] Besides, eledoisin and its congeners are hydrolyzed both by trypsin and chymotrypsin.

The prostaglandins are also hypotensive and they too relax the rat duodenum, but do not contract the cat jejunum muscle.[7] Moreover, the extract was previously defatted and, by chromatography on Bio-Gel P-2 columns, it was possible to calculate an approximate molecular weight of bufokinin which was much larger than the weight of prostaglandins. The presence of atropine and antihistaminic in the isolated organ perfusion solutions rules out acetylcholine and histamine, respectively, as possible hypotensive contaminants of the extract.

The importance of the neurohypophysial hormones, the skin, and the urinary bladder to the water economy of anurans has been well established.[15] Amphibians, frequently subjected to an alternating dry and aquatic environment, may be exposed to variations of its internal medium with the risk of great oscillations in the secretion of neurohypophysical hormone. This could lead to an overshoot in the permeability of the skin and the bladder unless there were a modulator to dampen the oscillations and excesses in neurohypophysial activity, hence allowing a more delicate adjustment of the homeostatic response of the amphibian organism. From a teleological point of view the kinins, by specifically and reversibly antagonizing the neurohypophysial hormones in the skin and in the urinary bladder of anurans, could be those physiological homeostatic modulators. The occurrence of kinin-forming and -destroying enzymes in the bladder would provide both a supply and a control of the kinin activity. When the animal moves from a terrestrial to an aquatic environment, the process of adjusting the body fluids to a new osmotic equilibrium might result in some dilution of the internal medium which, in turn, might conceivably stimulate the kininogenase activity of special tissues (e.g. the urinary bladder). The kinin liberated would thus inhibit the neurohypophysial activity, thereby helping to prevent overshoots in membrane permeability.

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